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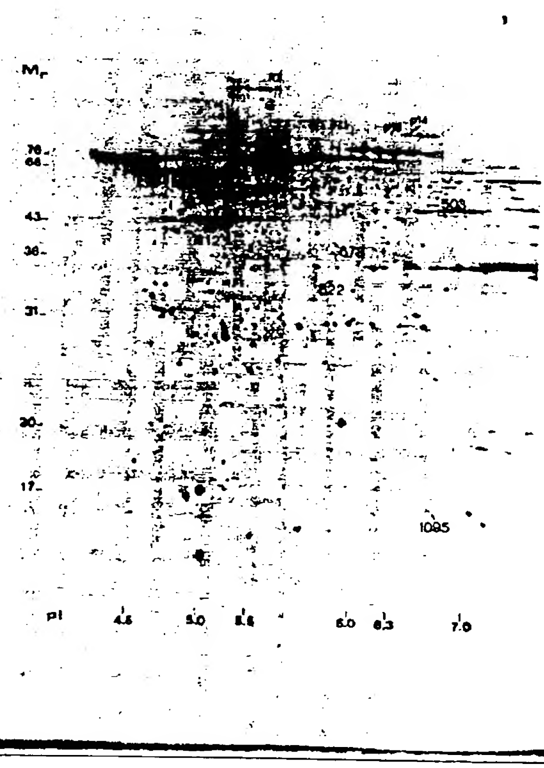
(54) Title: METHODS FOR THE DIAGNOSIS OF ALZHEIMER'S DISEASE

(57) Abstract

A method for the diagnosis of Alzheimer's Disease is disclosed. The method utilizes a unique set of proteins which are found to be altered in concentration in patients with Alzheimer's. The invention also relates to these proteins and their antibodies. Kits which can be used for the diagnostic test are also disclosed.



METHODS FOR THE DIAGNOSIS OF ALZHEIMER'S DISEASE

Field of the Invention

The present invention relates to methods for the diagnosis of Alzheimer's Disease and determination of effectiveness of treatment of Alzheimer's Disease. The methods involve measuring certain proteins in a body fluid or tissue sample which are found to be increased or decreased in concentration in patients with Alzheimer's Disease. The invention also relates to these certain proteins and antibodies to these proteins, and to diagnostic test kits.

Background of the Invention

Alzheimer's Disease (AD) is a major cause of presenile and senile onset dementia, affecting as many as 10 to 15% of the people over the age of 65. Alzheimer's Disease is a progressive dementia, characterized by degeneration of nerve cells in the cerebral cortex, presence of neuritic plaques and neurofibrillary tangles.

Early symptoms of Alzheimer's Disease differ widely from patient to patient, but commonly include psychological symptoms such as depression, paranoia and anxiety. There is a slow disintegration of personality and intellect. Along with the cognitive dysfunction, specific disturbances of speech (aphasia), motor activity (apraxia), and recognition of perceptions (agnosia) are observed. As the disease progresses, memory impairment increases.

The diagnosis of Alzheimer's Disease is a difficult one. There are many disease states which exhibit the same clinical symptoms as Alzheimer's Disease, including dementia secondary to treatable conditions such as infectious diseases and thyroid dysfunction, as well as psychiatric disorders. Currently, a definitive diagnosis of Alzheimer's Disease requires histopathologic

examination at autopsy. A Work Group on the Diagnosis of Alzheimer's Disease has established criteria for the clinical diagnosis of the disease. [McKhann et al., "Clinical Diagnosis of Alzheimer's Disease: Report of the NINCDS-ADRDA Work Group Under the Auspices of Department of Health and Human Services Task Force on Alzheimer's Disease", Neurology, Vol. 34, pp. 939-944 (July 1984)]. While this report of the Work Group attempted to refine the clinical diagnostic criteria for Alzheimer's Disease, there are to date no laboratory tests which would give a definite diagnosis of the disease.

Deficits in odor detection and discrimination are among the signs of Alzheimer's, and anatomical studies have suggested that olfactory pathways may be involved early on in Alzheimer's Disease. Pearson et al., Proceedings of the National Academy of Science, USA, Vol. 82, pp. 4531-4534 (1985). Recently, methods have been developed for culturing neuronal cells of adult human olfactory epithelium from living or deceased patients. Wolozin et al., Journal of Molecular Neuroscience, Vol. 3, pp. 137-146 (1992), incorporated herein by reference. Previous studies have shown that epithelium tissue taken at autopsy exhibits certain pathological changes in morphology, distribution and immunoreactivity of neuronal structures in patients with Alzheimer's Disease. Talamo et al., Nature, Vol. 337, pp. 736-739, (1989). In view of the fact that a large percentage of the elderly population is affected by Alzheimer's Disease, and because 20% or more of cases with a clinical diagnosis of Alzheimer's Disease are found at autopsies to have conditions other than Alzheimer's Disease, an objective laboratory test for the diagnosis of the illness is greatly needed.

Summary of the Invention

Accordingly, it is an object of the present invention to provide a laboratory test for Alzheimer's Disease.

It is another object of the present invention to

provide a method for determining the therapeutic effects of agents for the treatment of Alzheimer's Disease.

Various other objects and advantages of the present invention will be apparent from the drawings and the following description of the invention.

In one embodiment, the present invention relates to obtaining a definitive diagnosis of Alzheimer's Disease by observing changes in the concentration of certain proteins in body fluids or tissues from patients suspected of having Alzheimer's Disease as compared to normal controls.

In another embodiment, the present invention is directed to a method of determining the effectiveness of a course of treatment for Alzheimer's Disease for its ability to reverse or eliminate the disease, by observing a change in protein concentration of certain proteins in a body fluid or tissue sample to normal levels.

In a further embodiment the present invention relates to certain proteins which have been found to be increased or decreased in concentration in body fluid or tissue samples from patients with Alzheimer's Disease as compared to normal controls. Antibodies to these proteins, monoclonal or polyclonal, are contemplated in the present invention.

Finally, the present invention relates to a diagnostic test kit, which can be used in the clinical laboratory for the diagnosis of Alzheimer's Disease.

Brief Description of the Drawings

Figure 1: Silver-stained, two-dimensional gel of olfactory neuroblast total protein (40 μg) indicating five proteins that were found to be altered in relative amount when comparing 6 AD and 6 control cell lines (proteins 503, 612, 673, 822 and 1095). Relative molecular weight (y-axis) and isoelectric point (x-axis) values are indicated. Phosphoproteins were identified on autoradiograms of 2D gels by incorporation

of ^{32}P and are indicated on Figure 1 with a "p" prefix. None of the five AD-associated proteins were identified as phosphoproteins.

Figure 2: Graphs showing comparison of relative concentration values for five AD-associated proteins in olfactory neuroblasts from 8 control and 6 AD donors. Protein concentration values are reported as the percent total integrated density (% TID) of the protein of interest in comparison to all other proteins on the gel (y-axis). All samples were electrophoresed and analyzed in duplicate with similar results. "*" indicates that $p < 0.05$, "**" indicates $p < 0.01$, and "***" indicates $p < 0.005$.

Detailed Description of the Preferred Embodiments

The present invention relates to the identification of certain proteins which have been found to be present in increased or decreased amounts in cell cultures of olfactory neuroblasts of patients that had been diagnosed as having Alzheimer's Disease. It is contemplated that these proteins will also found at increased or decreased levels in body fluids and tissues of patients suspected of having Alzheimer's Disease. It is contemplated that the increased or decreased levels of these proteins may be detected by any test which can measure separate proteins in a body fluid or tissue sample, providing an objective test to definitively diagnosis Alzheimer's Disease. Any test which can measure the amount or concentration of separate proteins in the sample can be used in the present invention. In particular, immunological tests and electrophoresis are contemplated as useful in the present invention, although the invention is not limited to these procedures.

In another embodiment of the present invention, the

effectiveness of a course of treatment for Alzheimer's Disease can be determined by observing these proteins for proportional decreases or increases in levels to those of normal controls.

In developing this invention, the present inventors obtained cultures of neuronal cells of adult human olfactory epithelium from Alzheimer's Disease donors and age-matched controls according to known methods (Wolozin et al. supra). In vivo, olfactory neurons from patients suffering from Alzheimer's Disease (AD) develop AD-associated neurofibrillary pathology [Wolozin et al., Journal of Neuroscience Research, Vol. 33, pp. 163-169 (1992)]. Continuous cultures of AD-olfactory neuroblasts also exhibit elements of AD brain pathophysiology, including abnormal processing of amyloid precursor protein and an increase in potentially amyloidogenic C-terminal derivatives [Talamo et al., supra, and Wolozin (In press)]. The combination of the ability of olfactory neuroblasts to proliferate in cell culture and to express pathophysiological elements of AD makes these cells an ideal tool for the study of Alzheimer's Disease.

Using two-dimensional gel electrophoresis, the present inventors have identified five unique proteins that are altered in concentration when comparing olfactory neuroblast cell cultures from AD patients to controls.

The five proteins detected ranged in molecular weight from approximately 17kd to approximately 50kd, and all were acidic in varying degrees with pI's ranging from 4.8 to 6.7. Four of the five proteins (proteins 503, 612, 673 and 822) were increased at least 4-fold in olfactory neuroblasts from AD-donors, with two of the proteins being not present in normal control cells (proteins 673 and 822). One of the proteins (protein 1095) was decreased 2.5-fold in the cells from patients with Alzheimer's disease as compared to normal controls. The cell lines analyzed were matched for both age of donor, passage number and cell viability. Hence, the differences detected in these proteins appear to be related to the

only clearly different variable, the effects of AD.

The identity of these proteins is unknown, but a search of the SWISS-PROT Databank revealed several possible candidates based on pI and molecular weight values, including the extracellular signal related kinase 1 and 2 (ERK-1 and 2) (each about 45kd, and about pI 6.5) and the apolipoprotein E precursor (about 36kd, pI of about 5.7) which have both been associated with Alzheimer's Disease [Drewes et. al., EMBO, Vol. 11, pp. 2131-2138 (1992); and Strittmatter et al., Proceedings of the National Academy of Sciences, USA, Vol. 90, pp. 1977-1981 (1993)].

Altered protein phosphorylation has been implicated in the pathogenesis of Alzheimer's Disease [Saitoh et al., Alzheimer's Disease and Related Disorders, pp. 769-780 (1989)]. In order to determine whether alterations in phosphorylation contributed to the changes seen with the five AD-related proteins observed by the present inventors, olfactory neuroblasts were metabolically labeled with ^{32}P-orthophosphate. Subsequent analysis by 2D gel electrophoresis showed that none of the disease-related proteins were phosphorylated suggesting that increases in phosphorylation state did not directly account for the disease-related differences in the five proteins detected.

The lysosomal system has also been implicated in the pathogenesis of Alzheimer's Disease. Lysosomal catabolism appears to be necessary for constitutive production of ß-amyloid, and blockade of lysosomal catabolism in Alzheimer olfactory neuroblasts elicits large disease-related differences in amyloid precursor protein [Wolozin, supra, (In press); Haas, Nature, Vol. 359, pp. 322-324 (1992)]. The effects of lysosomal blockage on the five proteins detected by the present inventors were investigated. No significant changes in the levels of these five proteins were detected however. This suggests that, unlike ß-amyloid, these proteins were not products of lysosomal catabolism.

While the diagnosis of Alzheimer's Disease may be made on observing altered protein concentrations in the olfactory neuroblasts directly, such a method may not be practical for reasons of inconvenience to the patient, time and cost. It is therefore contemplated that body fluid or tissue samples obtained from a patient will be used to test for altered concentrations of these proteins for the diagnosis of Alzheimer's Disease. The body fluid examples include blood, serum, plasma, urine, cerebrospinal fluid and nasal mucosa. Most preferably, the body fluid sample will be cerebrospinal fluid, because it is generally accepted that proteins produced by nerve cells will be present in cerebrospinal fluid. However, it may be necessary to concentrate the cerebrospinal fluid by methods known in the art before subjecting it to an immunoassay or gel electrophoresis in order to obtain a similar protein concentration as that of the cell sample and thus detectable levels of the five proteins of this invention. In addition, immunoaffinity chromatography can be used to concentrate the proteins of interest in this invention in the CSF. Briefly, small amounts (eg., < 5 ml) of CSF would be loaded onto an immunoaffinity column containing either monoclonal antibodies or affinity-purified polyclonal antibodies covalently attached to the solid-phase matrix. The affinity column can consist of one or more antibodies made to the disease specific olfactory neuroblast proteins. After washing away contaminating molecules, the bound antigen(s) could then be detected immunologically, for instance by Western blot, RIA, ELISA or sandwich immunoassay. In addition, probes to these proteins, for instance specific monoclonal antibodies or nucleic acid probes, can be developed using cloning, PCR or immunologic methods to facilitate detection.

An example of a tissue sample useful in the diagnostic tests of the present invention is skin, which can be subjected to the test directly or the skin fibroblasts can be grown in tissue culture (by methods

well known in the art) prior to being tested.

The spots on the 2D gels can be viewed by any number of methods, including staining with Coomassie blue and silver staining. They can be visualized for relative protein density manually, but it is preferred that they be scanned with an appropriate camera system with a normalization standard and analyzed with a computer densitometer to measure relative protein spot staining intensities or densities.

The type of body fluid or tissue used as the sample will affect the two-dimensional gel pattern, although the five proteins of abnormal concentration found by the present inventors in the olfactory neuroblasts should appear at roughly the same relative molecular weights and pI's. Rather, it is the entire gel pattern which changes from one type of sample to another. For instance, one of skill in this art knows that two-dimensional gel patterns appear different in a sample of plasma as compared to a lymphoblast cell culture sample. While the present inventors have located the abnormal concentration proteins in gels from neuroblasts according to landmarked proteins on those gels, it is also possible to locate the same spots on a gel from a different type of sample even though in certain instances it may be necessary to refer to different landmark proteins. Ultimately, however, it is considered that a more precise method of identifying the proteins is by performing a Western blot on the gels from different body fluid or tissue samples, using antibodies which have been raised to the proteins of interest.

It is also understood that the present invention is not limited to the five proteins found so far, but to any proteins appearing in abnormal concentration in patients with Alzheimer's disease. Thus, different types of samples obtained from patients may show other proteins which are altered in concentration or, more importantly, may provide a more recognizable concentration difference on the two-dimensional gel pattern, perhaps by visualization with the naked eye, providing a simpler way

to score the sample as positive or negative.

In order to perform immunological tests for the diagnosis of Alzheimer's Disease, the first step is to obtain antibodies to the proteins of interest. There are many methods of accomplishing this which are well known to those skilled in the art. [For comprehensive laboratory methods, see Harlow et al., Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory (1988), which is incorporated by reference herein.] For antibodies with sufficient specificity for Western blots and immunoassays, the antigen must be purified to homogeneity or the antigen should be used to prepare monoclonal antibodies. Since the proteins of interest in this invention are seen as unique spots on the second dimension polyacrylamide gel, preferable the gel can be used as the final purification step of the individual antigens. One can obtain a pure antigen preparation by excising the spots which show increased or decreased intensity in Alzheimer's Disease patients. This gel piece can be injected into an animal to raise antibodies. Alternatively, one may cut out the spot of protein and electroelute it from the gel to obtain a protein in solution for injection. Still another technique for processing the protein for injection after separation on gels is to electrophoretically transfer the proteins to nitrocellulose, locate the proteins of interest by staining (e.g., with Ponceau S) excise the spots and cut them into pieces for injection. The particular method used is only limited by the ability to illicit an immune response to the proteins of interest.

As an alternative to using the antigen purified by separation on an electrophoretic gel directly, one may elute a protein spot, obtain a partial sequence by any method well known in the art, and use the sequence to manufacture synthetic peptides (usually with an automated machine using solid-phase techniques). If sufficient amounts of protein for partial sequencing are necessary, any of several methods can be used to obtain higher concentrations. For instance, the AD-specific proteins

can be purified using preparative two-dimensional electrophoresis techniques involving liquid-phase isoelectric focusing in combination with preparative polyacrylamide gel electrophoresis (Sanchez et al., Bio-Rad Bulletin 1744) or immobilized pH gradients in the first dimension [Strahler and Hanash, Methods: A companion to Methods in Enzymology, Vol. 3, pp. 109-114 (1991)]. Both of these techniques allow for at least a 100-fold purification of proteins from complex mixtures. The synthetic peptide should be at least six amino acids long to elicit antibodies that bind to the original protein. The purified synthetic peptides would then be coupled to carrier proteins and these conjugates would then be used to immunize animals.

The polyclonal antibodies in the antisera obtained with the foregoing methods can be used for Western blots and other immunological tests. However, one may further utilize hybridoma technology to obtain monoclonal antibodies, which may be the best choice for immunochemical techniques. Methods of monoclonal production are well known in the art and were first described by Kohler and Milstein in 1975. Briefly, antibody-secreting cells are fused to, for example, myeloma cells to create hybridoma cells which are cloned and screened by appropriate methods for the desired antibodies.

Any of the known immunological tests can be used for either detecting the presence or absence of a particular protein of interest, or to quantitate antigen concentration. An example of one assay is the two antibody sandwich assay described in Ghandbari et al., J. Clin. Lab. Anal., Vol 4, pp. 189-192 (1990). Briefly, in this assay two antibodies that bind to overlapping epitopes of the same antigen are combined in a sandwich enzyme-linked immunoassay. The antibodies can be either two monoclonals recognizing discrete sites or affinity purified polyclonals. One antibody is bound to a solid phase, antigen is bound, non-specifically bound proteins

are washed away, and a labeled second antibody is bound. The assay is quantitated by measuring the amount of labeled second antibody bound to the matrix. Many other immunoassays are well-known in the art and are useful in the present invention.

For purposes of illustrating the preferred embodiments of the present invention, the following examples are presented, it being understood that the present invention is not being limited by the examples.

EXAMPLE 1

Cell Culture

Olfactory neuroblasts were generated as described in Wolozin et al., Journal of Molecular Neurosciences, Vol. 3, pp. 137-146 (1992), incorporated herein by reference. Biopsies of olfactory epithelium were obtained from five neuropsychologically evaluated Alzheimer's donors (mean age 67.7 +/- 6.5 years) and seven age-matched controls (mean age 73.5 +/- 6.3 years). See Table 1. All samples were from subjects who had been evaluated by medical and neuropsychological testing. Diagnosis of Alzheimer's Disease was based upon the NINCDS-ADRDA criteria for "probable Alzheimer's Disease" (McKhann et al., supra).

To generate the cell lines of olfactory neuroblasts, samples of olfactory epithelium were imbedded in reconstituted basement membrane (Collaborative Research) and incubated in 4506 medium. The 4506 medium is a Hams-F12 based medium (neuroblast formulation, Whittaker), supplemented with 6% fetal calf serum (GIBCO), 150 μg/mℓ insulin (Sigma), 40 pg/mℓ thyroxine, 2.5 ng/mℓ selenious acid and 60 μg/mℓ gentamicin (Whittaker). After approximately three weeks, olfactory neurons grew from the tissue. Colonies of olfactory neurons with characteristic morphology were isolated using cloning cylinders and expanded in culture. All cell lines analyzed were between passage 7-10. Trypan blue exclusion analysis indicated that the cell lines were all viable and healthy.

To identify phosphoproteins by metabolic labelling of olfactory neuroblasts, cultures of ON cells were grown to

confluence in 60 mm culture dishes preincubated for one hour in phosphate-free Dulbecco's Modified Eagle Medium (DMEM;GIBCO) and then replaced with 0.5 mCi of ^{32}P orthophosphate (New England Nuclear) in 1.5 mℓ of phosphate-free DMEM. The cultures were incubated overnight and then harvested with a twenty minute incubation at 4°C in a buffer containing 100 mM TRIS, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/mℓ leupeptin, 10 μg/mℓ pepstatin, 10 μg/mℓ aprotinin and 10 μg/mℓ phenylmethylsulfonylfluoride. Levels of protein-incorporated radioactivity were determined by counting trichloracetic acid-precipitable counts in a scintillation counter.

Treatment of olfactory neuroblasts with the lysosomal inhibitor chloroquine results in the altered processing of the AD-associated amyloid precursor protein. To determine whether the five disease-related proteins were products of lysosomal degradation, olfactory neuroblasts were treated with the lysosomal inhibitor chloroquine. Olfactory neuroblasts were grown to confluence in 60 mm dishes in 4506 medium and then treated with 60 mM chloroquine overnight. Previous studies have shown that the cells survived this treatment without toxicity [Wolozin et al., Journal of Neuroscience Research, Vol. 33, pp. 163-169 (1992)].

Two-Dimensional Gel Electrophoresis

Olfactory neuroblast cells were harvested by scraping the dishes, adding protease inhibitors and sonicating. Samples containing equal amounts of total protein (40 μg) as determined by the method of Bradford [Analytical Biochemistry, Vol. 72, pp. 248 (1976)] were separated by high resolution two-dimensional gel electrophoresis according to the method of Hochstrasser et al. with minor revisions [Hochstrasser et al., Analytical Biochemistry, Vol. 173, pp. 424-435 (1989)]. In particular, total protein was prepared with the addition of a denaturing

solution containing 2% SDS, 2% CHAPS, 0.15 molar dithiothreitol (DTT), and 20% glycerol and heated at 95°C for four minutes. Proteins were separated in the first dimension by isoelectric focussing on polyacrylamide tube gels (3% T, 1% C) containing 9.2 molar urea, 4.35% pH 3-10 ampholines (Hoefer), 4.35% pH 4-8 ampholines (Hoefer), with diacrylpiperazine (PDA, Bio-Rad) as the crosslinking agent. Isoelectric focussing proceeded at 200 volts per hour, followed by 500 volts for five hours and 1200 volts for 10.5 hours. Separation of proteins in the second dimension was according to molecular weight on polyacrylamide slab gels containing 12% acrylamide, 0.2 molar TRIS-HCl pH 8.8, 0.7% (w/v) sodium thiosulfate, 0.3% (w/v) PDA, 0.5% (v/v) 1, 4-acrylpiperazine, and 0.07% (w/v) ammonium persulfate with a constant current of 40 ma per gel for approximately four hours. The resulting two-dimensional electrophoretograms were silver stained for computer analysis (see Hochstraser et al., supra, incorporated herein by reference). For the analysis of metabolically-labeled olfactory neuroblasts, equal amounts of protein-incorporated ^{32}P were separated on 2D gels and silver stained as described above. These gels were then dried and subjected to autoradiography.

Two-Dimensional Gel Analysis

Silver stained 2D gels of equal protein concentration from olfactory neuroblast samples were scanned with a Photometrics™ Series 200 CCD camera system and analyzed on a Sun Microsystems™ 4/260 computer with the Elsie program developed by Olson and Miller [Analytical Biochemistry, Vol. 169, pp. 49-70 (1988)]. This 2D gel analysis program uses an algorithm to distinguish polypeptide spots from background staining. Measurements were recorded as the difference in transmitted intensity, I_0-I, where I is the absorbed and I_0 is the unabsorbed incident light. Relative amounts of proteins are reported as the percent total integrated density (% TID) of the protein of

interest in comparison to all other proteins on the gel. All samples were electrophoresed and analyzed in duplicate with similar results.

Polypeptide spots that incorporated radio-labeled phosphate were identified as phosphoproteins on autoradiograms of silver stained 2D gels of total olfactory neuroblast protein. Phosphorylation state values of elongation factor-2 (EF-2) represent the ratio of the absolute TID values of the phosphorylated isoforms to the TID values of the nonphosphorylated isoforms as previously described [Johnson et al., Molecular Brain Research, Vol. 15, pp. 319-326 (1992)].

Statistical Analysis

Quantitative differences in relative amounts of each polypeptide based on Alzheimer's Disease and control % TID values were analyzed with Students' t-test, separate variance estimate. The individual sets for the five disease-related proteins were then analyzed for combined significance levels.

Olfactory neuroblasts from Alzheimer's Disease patients showed alterations in the relative amounts of five proteins when compared to controls. Computer-assisted analysis of olfactory neuroblast protein samples separated on 2D gels from six Alzheimer's Disease and six control patients revealed five polypeptides that were different in concentration to a statistically significant degree (Figure 1). Four of these proteins (proteins 502, 612, 673 and 822) were increased in relative amount in the Alzheimer's Disease cells while protein 1095 was increased in normal controls (i.e., decreased in AD samples). Table 2 summarizes the relative molecular weight and isoelectric point values for each of these polypeptides.

Analysis of metabolically-labelled proteins showed that none of the five polypeptides found to be altered in the AD cells incorporated ^{32}P, indicating that they were not phospoproteins (Figure 1). Two phosphoproteins at 97kd

(P13 and P14) had been previously identified as phosphorylated forms of elongation factor-2 (EF 2). Analysis of 2D gels from AD and control brain homogenates shows an increase in the phosphorylation state of EF 2 in Alzheimer's Disease-affected brain regions (Johnson et al., supra). However, the present inventors found no increase in the ratio of the TID values of the phosphorylated isoforms of EF 2 (P13 and P14) to the nonphosphorylated isoforms (the two more basic isoforms) when comparing olfactory neuroblasts from Alzheimer and control patients (0.74 +/- 0.24 for Alzheimer's Disease (n=6); 0.80 +/- 0.13 for a control, (n=6) (Figure 1).

In order to investigate the effect of the lysosomal system on the concentration of the proteins found to altered in AD cells, ON cell lines from AD and control subjects were treated with a lysosomal inhibitor, chloroquine. All five of the polypeptides showed differences in concentration values similar to those found in untreated cells when comparing samples from AD and control patients ($p < 0.05$ for differences in all five proteins). However, lysosomal blockage did not result in a significant enhancement of these differences.

Protein 673 appears in this study to be of particular interest for a diagnostic marker because of the lack of overlap between the AD and control samples. In addition to assessing the diagnostic value of the five disease-related proteins as independent data sets, also employed is a model where in order to have a diagnosis of AD, a subject must have a positive % TID value for more than one protein. Using this combinatorial model, the power of the test is equal to the product of the perspective p-values. An example of the increased significance using this criteria is shown when it is stipulated that AD is diagnosed by having positive % TID values for both proteins 216 and 673. In this case, the p value is increased from < 0.005 for each of the individual proteins to < 0.0001 for the two proteins in combination. It is then apparent that a diagnostic test taking into

consideration all five marker proteins in combination is particularly powerful for indicating whether a subject has Alzheimer's disease or not, where a diagnosis can be made on the basis of positive values for all five proteins. In general, the more positive results there are for the five proteins, the more definitive the diagnosis.

TABLE 1

Olfactory Neuroblast Donors

Cell Line	Age	Diagnosis
91-4	75	probable AD
91-5	66	probable AD
91-10	56	probable AD
91-12	71	probable AD
91-15	68	probable AD
91-19	70	probable AD
91-9	78	normal
91-14	69	normal
91-17	66	normal
92-18	69	normal
92-21	82	normal
92-22	77	normal

TABLE 2

Relative Migration Values of Proteins Altered in Olfactory Neuroblast Lysates from AD and Control Donors

Protein Number	M_r (kd)	pI
503	50	6.5
612	40	4.8
673	38	5.7
822	35	5.8
1095	17	6.7

EXAMPLE 2

To examine the effectiveness of a course of treatment for Alzheimer's Disease, a body fluid or tissue sample, preferably cerebrospinal fluid, would be obtained

from an Alzheimer's Disease patient prior to the treatment. A two-dimensional gel would be run, stained and analyzed for levels of one or more of the proteins listed in Table 2 to obtain a baseline. After instituting treatment, one or more body fluid or tissue samples are taken from the patient and analyzed for levels of the same protein or proteins which were initially analyzed. A proportional increase or decrease to normal levels (depending on whether the protein analyzed is one which is found to increase or decrease in Alzheimer's Disease patients) signifies that the treatment is successful.

EXAMPLE 3

A. Preparing Antigens

After two-dimensional gel electrophoresis is performed on a patient with confirmed Alzheimer's Disease, the gel would be stained with Coomassie blue in order to locate a protein of interest (for example, one of the five proteins in Table 2). The gel would be rinsed with deionized water for a few minutes, changing the water several times. The spot containing a protein would be cut out of the gel with a scalpel, and placed on a piece of parafilm or plastic wrap. The edge of a paper towel is used to remove any standing water by capillary action. Next, the plungers from the barrels of two 5 cc syringes are removed and the gel piece is placed into one of the barrels. The plunger is then replaced and the syringe outlet is positioned in the barrel of the second syringe. Using rapid, firm pressure on the plunger, the gel is pushed into the barrel of the second syringe. This process is repeated several times back and forth between the two syringes. Then, 21-gauge needles are placed onto the outlet of the syringes, and the process is repeated. A small amount of buffer (PBS) may be necessary to keep the small fragments passing back and forth between the syringes. The samples are now ready for injection.

B. Preparing Antisera

Antibodies are raised in rabbits immunized by injecting the antigen preparation (above). An initial subcutaneous injection of approximately 150 μg of one of the protein preparations would be followed by two monthly injections of approximately 100 μg of the antigen. This will lead to a sufficient antibody titer for use in an immunoassay.

C. Preparing Monoclonals

Monoclonal antibodies may be prepared according to the method of Kohler and Milstein. This method involves immunizing mice with an antigen bearing one or more epitopes (i.e., one of the Alzheimer's Disease proteins of Table 2). The mice develop spleen cells making anti-epitope (SN) which appear as an antibody (or antibodies) in the serum. The spleen is removed and the individual cells fused in polyethyleneglycol with constantly dividing (i.e., immortal) B-tumor cells selected for a purine enzyme deficiency and often for their inability to secrete Ig. The resulting cells are distributed into microwell plates in HAT (hypoxanthine, aminopterin, thymidine) medium which kills off the profusion partners, at such a high dilution that, on average, each well will contain less than one hybridoma cell. Each hybridoma being the fusion product of a single antibody-forming cell and a tumor cell will have the ability of the former to secrete a single species of antibody and the immortality of the latter enabling it to proliferate continuously, clonal progeny providing an unending supply of antibody. The hybridomas which secrete the monoclonal antibody of interest are screened by using a solid phase assay with an enzyme reaction product which provides a positive signal, i.e., an ELISA. For the ELISA, the antigen of interest is bound to a solid support, such as a 96 well plate. Then a sample of the culture medium from each of the hybridomas is placed in the well containing the bound antigen. The medium containing the antibody to the antigen of interest will

react with that antigen which has been bound to the plate. Unbound antibody is washed away and a second antibody which reacts with the mouse antibody, and which binds to the hybridoma antibody already bound to the antigen, is added. This second antibody contains an enzyme tag, such as peroxidase or alkaline phosphatase. By then adding the appropriate substrate, the enzyme produces a colored reaction product which may then be observed visually or through the use of a machine that can measure color intensity. Once positive hybridomas are found through the screening procedure, the hybridomas are cloned so that eventually a pure culture of a single hybridoma line which produces the antibody of interest is obtained, providing an unlimited supply of the monoclonal antibody.

EXAMPLE 4

Typical test kits for use with a radioimmunoassay or ELISA test will contain:

(A)

1. A plate with absorbed rabbit FAB fragment IgG (to any of the proteins set forth in Table 2), or nitrocellulose sheets with the absorbed rabbit IgG.

2. Rabbit whole IgG (to the same protein as above).

3. Labelled goat anti-rabbit IgG (F_c portion).

(B)

1. Mouse monoclonal (to any of the proteins listed in Table 2).

2. Labelled goat anti-mouse.

These kits may also contain appropriate buffers such as PBS, blocking solution and appropriate enzyme substrates (for ELISAs). These materials may be provided with the kit or may be separately provided or prepared.

The term "plate" is used in the broad sense to include any flat surface which can be employed with an RIA or ELISA. In practice, the test kit A (above) would be employed as follows:

1. Incubate the plate with the serum of the

patient under test for an appropriate time and temperature (e.g., from 2 to 4 hours at 37°C).

2. Wash with BSA/PBS.

3. Incubate with rabbit whole IgG and wash with buffer.

4. Incubate with labelled goat anti-rabbit IgG (F_c portion) and wash with the same buffer.

5. Detect the formation of a reaction product (or radioactive signal) in the case of a positive test by any of the known procedures.

The test kit B would be employed as follows:

1. Incubate a substrate (plate, nitrocellulose paper, etc.) with an unknown sample (such as cerebrospinal fluid) for an appropriate time and temperature.

2. Wash with BSA/PBS.

3. Incubate the substrate with the mouse monoclonal and wash with buffer.

4. Incubate with goat anti-rabbit IgG and wash with same buffer.

5. Detect the formation of a reaction product (or radioactive signal) in the case of a positive test by an appropriate procedure.

CLAIMS

1. A method of diagnosing Alzheimer's Disease, comprising:

subjecting a body fluid or tissue sample from a patient suspected of having Alzheimer's Disease to a test which measures separate proteins in the body fluid;

determining a protein or proteins which are present in increased or decreased concentration in said sample as compared to a control sample from a normal subject,

wherein said increase or decrease in concentration is indicative of a positive diagnosis for Alzheimer's Disease.

2. The method of claim 1, wherein the test is an immunological test or electrophoresis.

3. The method of claim 1, wherein the protein which is increased in concentration is at least one selected from the group consisting of those proteins designated by the spots numbered 503, 612, 673 and 822 in Figure 1.

4. The method of claim 3, wherein the proteins which are increased in concentration are at least two of the proteins designated by the spots numbered 503, 612, 673 and 822.

5. The method of claim 4, wherein the proteins which are increased in concentration are 503 and 673.

6. The method of claim 1, wherein the protein which decreases in concentration in patients with Alzheimer's disease is the protein designated by the spot numbered 1095 in Figure 1.

7. The method of claim 2, wherein the

electrophoresis is two-dimensional gel electrophoresis and protein concentration is obtained by measuring stain intensity of spots on a gel stained with Coomassie blue or silver.

8. The method of claim 1, wherein the body fluid is selected from the group consisting of serum, plasma, cerebrospinal fluid, urine and nasal mucosa.

9. The method of claim 8, wherein the body fluid is cerebrospinal fluid.

10. The method of claim 1, wherein the tissue sample comprises fibroblasts.

11. A method of diagnosing Alzheimer's Disease, comprising:

subjecting a body fluid or tissue sample from a patient suspected of having Alzheimer's Disease to a test which measures separate proteins in the body fluid or tissue; and

detecting the presence, as compared to a control sample from an individual without Alzheimer's Disease, of protein 673, wherein the presence of said protein confirms that said patient has Alzheimer's Disease.

12. The method of claim 11, wherein the test is an immunological test or electrophoresis.

13. The method of claim 11, wherein the body fluid sample comprises cerebrospinal fluid.

14. The method of claim 11, wherein the tissue sample comprises fibroblasts.

15. The method of claim 11, wherein the

presence of said protein is detected by a Western blot technique.

16. A method of determining the effectiveness of treatment for Alzheimer's Disease comprising:

measuring the concentration of a protein or proteins in a body fluid or tissue sample from a patient with Alzheimer's Disease prior to initiating treatment; and

measuring the concentration of the same protein or proteins of a same body fluid or tissue after treatment has been instituted,

wherein a proportional decrease or increase of protein to levels found in an individual without Alzheimer's Disease indicates the effectiveness of treatment.

17. The method of claim 16, wherein the tissue sample comprises fibroblasts.

18. The method of claim 16, wherein the body fluid sample comprises cerebrospinal fluid.

19. The method of claim 18, wherein said cerebrospinal fluid is subjected to two-dimensional electrophoresis or an immunoassay.

20. The method of claim 19, wherein the two-dimensional gels are stained by silver-staining or staining with Coomassie blue.

21. The method of claim 20, further comprising quantifying the concentration by measuring the stained density with a densitometer.

22. The proteins designated by the spots numbered 503, 612, 673, 822, and 1095 in Figure 1.

23. Antibodies to the proteins designated by the spots numbered 503, 612, 673, 822, and 1095 in Figure 1.

24. The antibodies of claim 23, which are monoclonal.

25. A test kit for detecting Alzheimer's Disease, said kit comprising:

a diagnostically effective amount of an antibody to one of the proteins selected from the group consisting of: 503, 612, 673, 822 and 1095;

a labelled antibody to said antibody.

26. The test kit of claim 25, wherein said antibody is polyclonal or monoclonal.

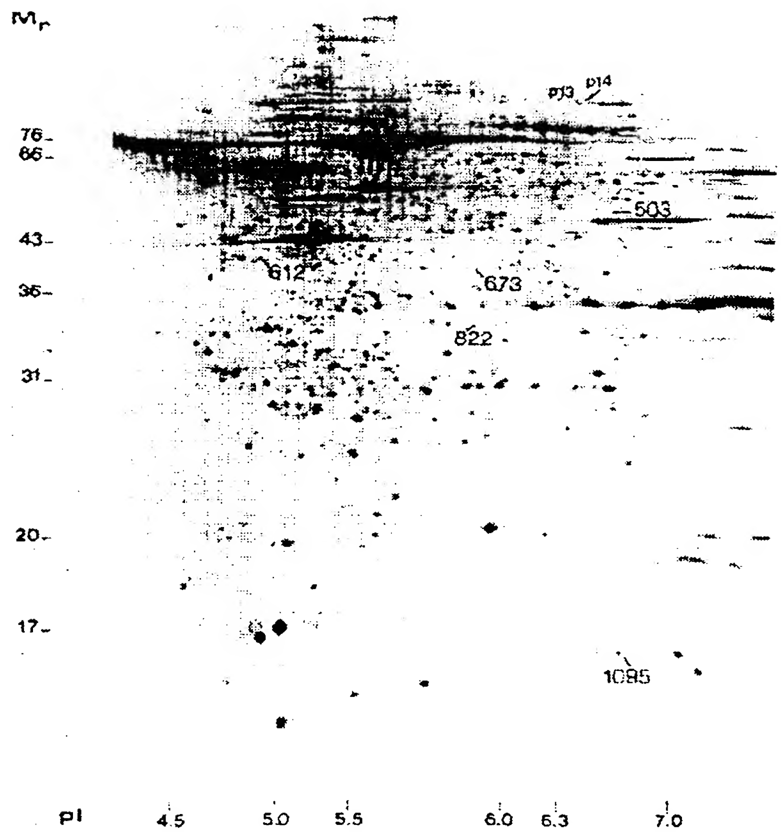

Mr
76
66
43
36
31
20
17
p13
p14
503
612
673
822
1085
pI
4.5
5.0
5.5
6.0
6.3
7.0

Fig. 1

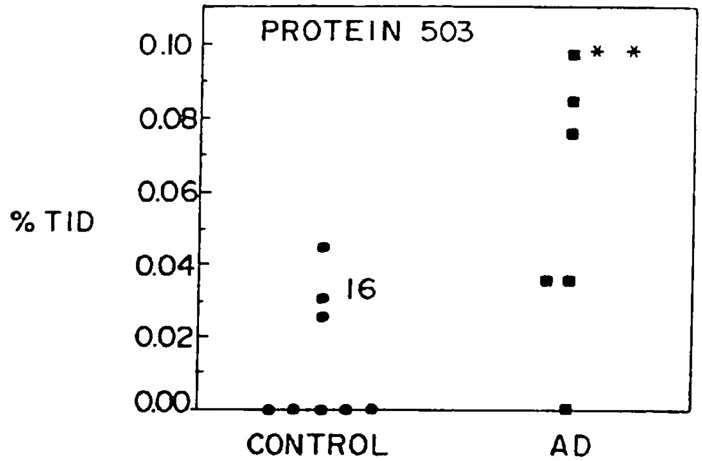


Fig. 2(a)

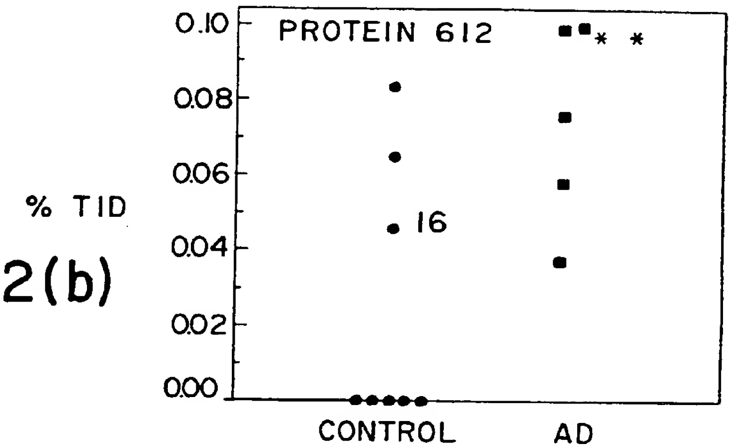


Fig. 2(b)

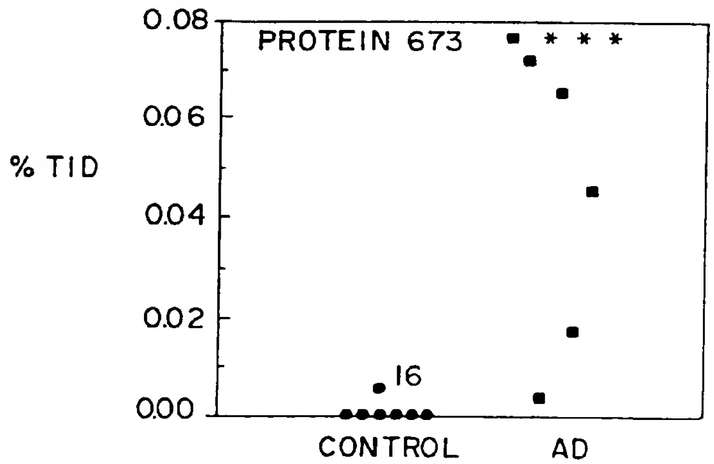


Fig. 2(c)

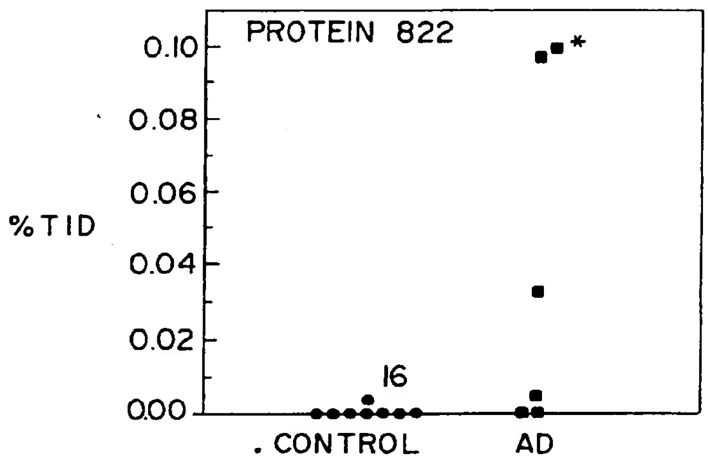

PROTEIN 822
%TID
0.10
0.08
0.06
0.04
0.02
0.00
16
*
CONTROL
AD

Fig. 2(d)

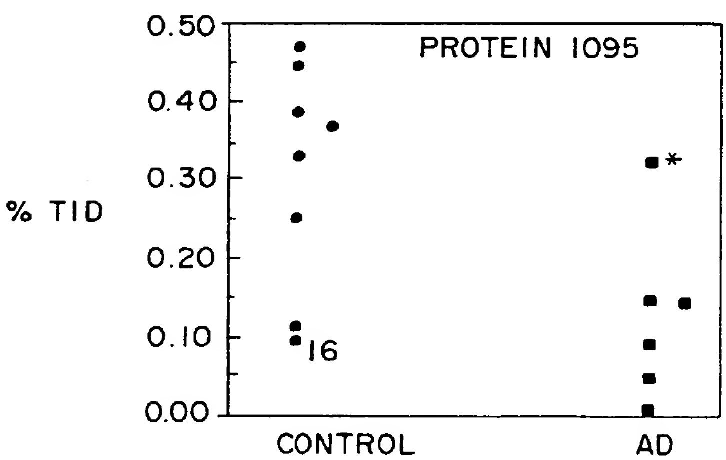

PROTEIN 1095
% TID
0.50
0.40
0.30
0.20
0.10
0.00
16
*
CONTROL
AD

Fig. 2(e)